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FOREWORD

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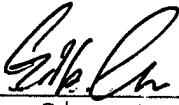
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INTRODUCTION

Significance of double-strand breaks

DNA double-strand breaks (DSBs) may be the most disruptive form of DNA damage. If left unrepaired, they lead to broken chromosomes and cell death. If repaired improperly, they can lead to chromosome translocations and cancer. Humans are at risk for DSBs from exogenous agents. The paradigm agent, ionizing radiation (IR), is present in the environment from the decay of radon and radon daughters, which accumulate to high levels in homes built on land reclaimed from mining, leading to increased cancer risk. Ionizing radiation can cause cancers in many tissues, with breast tissue being particularly sensitive (6, 12). Mice exposed to IR show a dose-dependent increase in breast cancer (59). Women exposed by the atomic bombs detonated over Hiroshima and Nagasaki (42) or treated with radiation for post partum mastitis (51) had increased risks for breast cancer.

DSBs are also created during the treatment of cancer by the use of IR or anticancer drugs, such as bleomycin, which produces oxidative free radicals, or etoposide and adriamycin, which inhibit topoisomerase II to create protein-bridged DSBs. Humans are also at risk for DSBs from endogenous agents. Oxidative metabolism generates free radicals and subsequent strand breaks. V(D)J recombination generates DSBs during rearrangement of genes encoding B cell immunoglobulins and T cell receptors (53).

In response to the threat of DSBs, cells have evolved at least two independent pathways for repairing DSBs, by homologous recombination or by nonhomologous DNA end-joining. We have chosen to focus on understanding the dominant mechanism in mammalian cells, nonhomologous end-joining, which has proven to be involved in both resistance to IR and V(D)J recombination.

Mutant cell lines

Immunological diversity is generated by V(D)J recombination, a site-specific cleavage of the chromosome followed by an end-joining reaction to bring the free DNA ends together (53). The possibility that DSB repair and V(D)J recombination might share the same biochemical pathway was first recognized in the severe combined immunodeficiency (*scid*) mouse. The *scid* mouse lacks mature B and T cells due to a defect in V(D)J recombination (31) and is also hypersensitive to IR due to a defect in DSB repair (2, 15, 22). Screening of IR sensitive cell lines led to the identification of four genetic complementation groups with defects in both DSB repair and V(D)J recombination (38, 56). Since efforts were underway to complement these rodent cell lines with human genes, the genes for the complementation groups were designated *XRCC*, for X-ray cross-complementing. *XRCC7* mutant cells (*scid* mouse cells and V3 hamster cells) were severely defective in coding joint formation but only mildly defective in signal joint formation. By contrast, *XRCC4*, *XRCC5*, and *XRCC6* mutant cells were severely defective in both coding and signal joint formation.

Ku and DNA-dependent protein kinase

Ku was originally identified as a major autoantigen in scleroderma-polymyositis overlap syndrome (34, 47). Ku is a heterodimer of 70 and 86 kDa (Ku70 and Ku86) that binds with strong affinity to DNA ends, stem-loop structures, or transitions between double-stranded DNA and two single strands (13, 35). Once Ku binds to DNA, it can translocate along the DNA in an ATP-independent manner, so that three or more Ku molecules can bind to a single linear DNA fragment (10, 37). Ku is also a DNA-dependent ATPase that is activated by both double and single-stranded DNA (7), and motifs for potential ATP binding sites are found in both Ku70 and Ku86. Ku has also been reported to have an ATP-dependent 3' to 5' helicase activity (58).

Ku is the regulatory subunit for DNA-dependent protein kinase (DNA-PK), which has the unusual property of being activated by DNA ends (19). DNA-PK contains an enormous catalytic subunit of 460 kDa (p460) that is activated when Ku binds to DNA. Interestingly, DNA-PK will confer ATPase activity to dephosphorylated Ku protein (7). However, attempts to identify *in vivo* substrates have been inconclusive. For example, we and others have shown that p460 is not required for the accumulation or activation of p53 (14, 46).

Genes for double-strand break repair

In searching for proteins that recognized altered DNA structures, we identified a DNA end-binding (DEB) activity in cell extracts, which bound specifically to DNA ends (44). A large number of IR-sensitive cell lines were screened, and DEB factor was absent in three different cell lines, all belonging to the complementation group for *XRCC5* (44). DEB factor proved to be both biochemically and antigenically similar to Ku (16, 45). To see if *XRCC5* cells are defective in Ku, we transfected expression vectors for Ku70 and Ku86 into the mutant *XRCC5* hamster cells. Transfection of human Ku86 rescued the mutant hamster cells for DEB activity, DNA-PK enzymatic activity, IR resistance, and V(D)J recombination (54, 55). These results demonstrated that Ku is involved in the repair of DNA DSBs produced by ionizing radiation or V(D)J recombination. We and others then showed that *XRCC5* cells contained mutations in the *Ku86* gene (11, 52), leading to functionally significant alterations in Ku86 (Table 1). As expected, the generation of *Ku86* knockout mice produced a severe combined immunodeficiency due to an absence of both T and B cells (36, 61), as seen for scid mice. Both coding and signal joint formation were impaired, and the coding joint defect was accompanied by accumulation of hairpin coding ends in *Ku86* knockout thymocytes.

The discovery that Ku was defective in *XRCC5* mutant cells raised the possibility that p460 might be defective in *XRCC7* cells (4, 24, 39). Indeed, DNA-PK enzymatic activity and p460 protein levels were severely reduced in both scid and V3 cells. Transfection of V3 and scid cells with genomic yACs carrying the p460 gene restored DNA-PK enzymatic activity, IR-resistance, and coding joint formation. Furthermore, the *p460* gene is mutated in scid cells, producing a premature termination codon in the putative kinase domain that truncates the C-terminal 83 amino acids (5, 9). As in *Ku86* knockout thymocytes, scid thymocytes accumulate hairpin coding ends (61). Therefore, Ku and DNA-PK are required for the proper processing of hairpin ends. Ku or DNA-PK could have an endonuclease activity that opens the hairpin ends directly, but such an endonuclease activity has not been reported. More likely, DNA-PK enzymatic activity makes the hairpin accessible to a still unidentified hairpin endonuclease.

The only known *XRCC4* cell line, XR-1, is rescued by transfection of a cDNA encoding a 37 kDa protein, restoring both IR-resistance and V(D)J recombination to wild type levels (29). *XRCC4* is a novel gene, lacking homology to any known gene in the current data base. It is deleted in XR-1 cells, and therefore not essential for growth. The sensitivity of XR-1 cells increases dramatically in the G1 phase of the cell cycle (17), and the proposed role for *XRCC4* in stimulating ligase activity (20) must account for this phenomenon.

Table 1. Proteins involved in double-strand break repair. Genetic complementation groups, corresponding to the X-ray cross complementing genes, *XRCC4* - *XRCC7*, have been assigned for cell lines defective in double-strand break repair and V(D) recombination. All cell lines are hypersensitive to IR. The *XRCC6* mutant cell line was generated by targeted knockout of mouse embryonic stem cells.

Mutated gene (protein)	V(D)J defect	Mutant cells	Mutation
<i>XRCC4</i>	coding/signal	XR-1	gene deletion (29)
<i>XRCC5</i> (Ku86)	coding/signal	XR-V9B	internal a.a. deletion (11)
		XR-V15B	internal a.a. deletion (11)
		xrs4	truncated 287 a.a. protein (52)
		xrs5	?
		xrs6	truncated 24 a.a. protein (52)
<i>XRCC6</i> (Ku70)	coding/signal	ES cells	knockout (21)
<i>XRCC7</i> (p460)	coding	scid	terminal 83 a.a. truncation (5, 9)
		V3	?

DNA end-joining in intact cells

In vivo properties of the end-joining reaction have been revealed by experiments with intact mammalian cells. When naked DNA was transfected into mammalian cells, end-joining either occurred by direct joining of the ends or by involving deletions back to regions of microhomology of 1 to 6 bases (49, 50). The latter joining events appeared to be directed by base pairing of the microhomology regions, thus aligning the DNA ends for subsequent steps in the end-joining reaction.

To study end-joining of chromosomal DNA, restriction enzymes have been used to introduce DSBs at specific chromosomal sites. Restriction enzymes were electroporated into Chinese hamster ovary cells, which are hemizygous at the *APRT* (adenine phosphoribosyl transferase) locus (41). Colonies of viable cells containing mutations in *APRT* were then analyzed at the DNA sequence level. Restriction enzyme-induced mutations consisted of insertions, small deletions up to 36 bp, and combinations of insertions and deletions at the cleavage sites. Most of the deletions involved the utilization of microhomology of 1 to 4 bases at the recombination junctions. Thus, the end-joining reactions observed with transfected naked DNA and chromosomal DNA shared key characteristics: nucleotide insertions and nucleotide deletions directed by microhomology. Furthermore, when chromosomal DNA was cleaved by a rare cutting restriction enzyme, the subsequent end-joining required Ku (30).

V(D)J recombination junctions provide additional insight into end-joining of broken chromosomes (53). Coding joint formation occurs with both insertion and deletion of nucleotides. Most insertions occur by mechanisms specific for lymphoid cells. Palindromic (P-nucleotide) insertions are generated by the asymmetric cleavage of the hairpin coding ends created by RAG1/RAG2 cleavage. N-nucleotide insertions are generated by the random addition of nucleotides catalyzed by lymphoid specific terminal deoxynucleotidyl transferase (TdT). Nucleotide deletion is also best studied in TdT knockout mice, since a high frequency of TdT-catalyzed insertions can obscure the nature of the deletions. In the absence of TdT, 75% of the joining events contain deletions directed by microhomology regions of 1 to 5 nucleotides (18, 25). Thus, the joining reaction for coding ends during V(D)J recombination confirms the characteristics observed for random DSBs in nonlymphoid cells. In contrast to coding joint formation, signal joint formation is precise and dependent on Ku, but not on an intact kinase domain in p460. Thus, the mechanisms that lead to the insertion and deletion of nucleotides are somehow suppressed during the formation of signal ends.

DNA end-joining in cell-free extracts

Progress towards defining nonhomologous end-joining in a cell-free system has been slow compared to progress in identifying the relevant genes. End joining was studied in *Xenopus* extracts by recircularization of linear DNA molecules (40, 57). In contrast to intact mammalian cells, *Xenopus* extracts did not produce nucleotide deletion beyond losses from protruding single strands. End-joining in mammalian extracts has been studied by the recircularization of plasmid DNA cleaved with restriction enzymes. Almost all events involved direct joining of the cohesive ends, but there were a small number of misrejoined molecules with insertions or deletions utilizing regions of microhomology. It was possible to fractionate extracts into four different end-joining activities (23). The activity that utilized microhomology near the ends could be purified extensively (32). However, none of the activities was dependent on the presence of Ku protein. Therefore, these cell free systems failed to detect the Ku-dependent end-joining mechanism in mammalian cells.

There has been success in obtaining DNA end-joining in cell-free extracts for the special case of V(D)J recombination (8, 43). Cleavage by RAG1 and RAG2 was followed by a rejoicing reaction with the appropriate properties: precise joining of the signal ends and utilization of microhomology for the coding ends, albeit with low efficiency. For the special case of signal end joining, the reaction appeared to be specific for Ku since it was inhibited by anti-Ku antibodies (8).

In summary, because of the limited progress in recovering DSB repair *in vitro*, the roles of Ku, DNA-PK, and XRCC4 remain poorly defined. Furthermore, additional proteins must be required for the reaction, and these proteins and their genes remain unidentified. This proposal will address these issues.

BODY**Publications from this laboratory related to this report**

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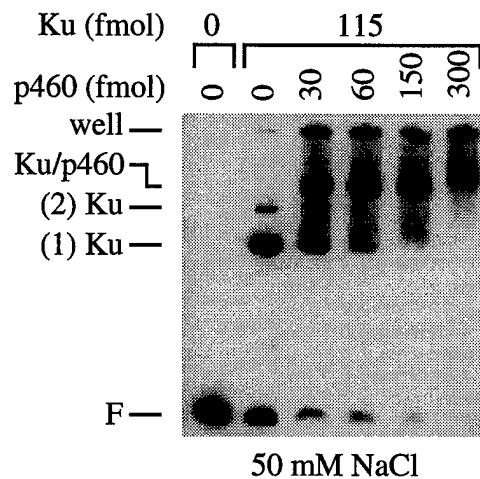
EMSA for assembly of Ku and p460 onto a DNA end

The work described in Background and Significance identified four genes involved in a common biochemical pathway for rejoicing free DNA ends shared by DSB repair and V(D)J recombination. To study this pathway biochemically, we previously developed an electrophoretic mobility shift assay (EMSA) for measuring Ku binding to DNA ends. This assay was extremely sensitive for Ku but failed to reveal the assembly of the full DNA-PK enzymatic complex on DNA ends. We therefore developed a new EMSA that would circumvent this problem. In the new EMSA, extracts were incubated with a short DNA probe of only 32 bp (f32). We discovered that the short DNA probe was not affected by nonspecific DNA binding proteins, so that supercoiled competitor DNA could be omitted from the binding reaction.

In the new EMSA, incubation of purified Ku with f32 probe DNA produced a major protein-DNA complex, corresponding to a single binding event of Ku to f32, and a minor complex, corresponding to two binding events of Ku to f32 (Fig. 1). These complexes were also observed in the old EMSA. Incubation of purified Ku and purified p460 with f32 produced two additional lower mobility complexes (Fig. 1). One complex corresponded to the binding of one Ku and one p460 molecule. A second complex was retained in the well of the gel and therefore corresponded to the binding of at least two Ku/p460 molecules. Although this complex may be a nonspecific aggregate, we plan to explore the possibility that it represents a well defined synapsis of two DNA molecules mediated by the association of two p460 molecules.

Incubation of f32 with crude HeLa extracts produced a series of complexes that comigrated with the complexes seen in Fig. 1. The identity of the complexes from the HeLa extracts was confirmed in several ways. First, three different monoclonal antibodies against different epitopes of p460 were added to the binding reaction and were found to either supershift or disrupt the complex that comigrated with the Ku/p460/DNA complex (V. Smider and G. Chu, manuscript in preparation). Second, when antibodies against Ku were added to the binding reaction, they supershifted the complexes that comigrated with the Ku/DNA and Ku/p460/DNA complexes. Third, when the f32 probe was incubated with extracts from V3 and scid cells, which are defective in p460 (see Table 1), the low mobility Ku/p460/DNA complex was specifically absent. Therefore the new EMSA provided a rapid assay for purifying Ku and p460 from crude extracts.

Figure 1. EMSA for assembly of DNA-PK on DNA. The 32 P-labeled f32 DNA probe was incubated with purified Ku plus different amounts of purified p460. After 10 min. at room temperature, the products were resolved by electrophoresis in nondenaturing polyacrylamide gel and then visualized by autoradiography. The position of the free DNA probe (F) is shown at the bottom of the gel. With Ku alone, two complexes were observed, corresponding to the binding of 1 and 2 Ku molecules to the DNA. With Ku and p460, new complexes were observed, one corresponding to the binding of 1 Ku and 1 p460 molecule to the DNA (Ku/p460) and one super-large complex that was retained in the well of the gel.



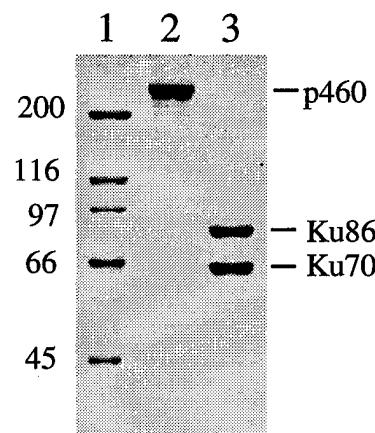
Purification of Ku and p460

To further study the biochemical roles of Ku and p460 in double-strand break repair, it was critically important to obtain highly purified preparations of both proteins. During the course of purification, we noted the presence of a complex that appeared to contain p460 in the absence of the specific Ku/DNA complex. We therefore developed a protocol to successfully purify Ku and p460 separately (Fig. 2).

In particular, the p460 preparation was freed of contaminating Ku by the use of an oligonucleotide affinity column consisting of 1 ml of NHS-agarose (HiTrap, Pharmacia) coupled to 0.8 mg of f32 according to the manufacturer's protocol. One oligonucleotide in the double-stranded f32 was substituted at the 3'-end with an amino group to allow efficient coupling to the NHS-agarose resin (26). The oligonucleotide column was then eluted with a 0.1 to 0.5 M NaCl gradient. Under these conditions, p460 eluted at 0.25-0.30 M NaCl, leaving the majority of Ku bound to the resin, since Ku normally eluted at 0.55-0.60 M NaCl. The trace amount of Ku contaminating the p460 fraction was removed by running the pooled p460 fraction through the oligonucleotide affinity column at 0.25 M NaCl. Under these conditions, p460 was collected in the flowthrough while the contaminating Ku and a minor fraction of p460 remained bound to the resin. The level of Ku contamination was measured both by immunoblot and EMSA. The molar ratio of Ku to p460 was between 1:2000 and 1:4000 by the use of serial dilutions.

Figure 2. SDS-PAGE of separately purified Ku and p460.

Ku and p460 were separately purified to homogeneity from human placenta by chromatographic methods. The preparations were then analyzed by SDS-PAGE followed by staining with Coomassie blue. The p460 preparation (lane 2) was freed of Ku contamination down to a molar ratio between 1:2000 and 1:4000 by using an oligonucleotide affinity column. The Ku preparation (lane 3) contained no detectable p460.



p460: DNA binding and activation in the absence of Ku

Previous reports suggested that Ku was required for both DNA binding and kinase activity (19). To our surprise, we discovered that in low salt buffer (10 mM NaCl), the highly purified p460 bound to f32 DNA in the EMSA, even in the absence of Ku. Two distinct p460/DNA complexes were observed: one that migrated slightly faster than the Ku/p460/DNA complex, and a second that was retained in the well of the gel, corresponding to a complex containing at least two p460 molecules. Furthermore, the binding was biochemically significant

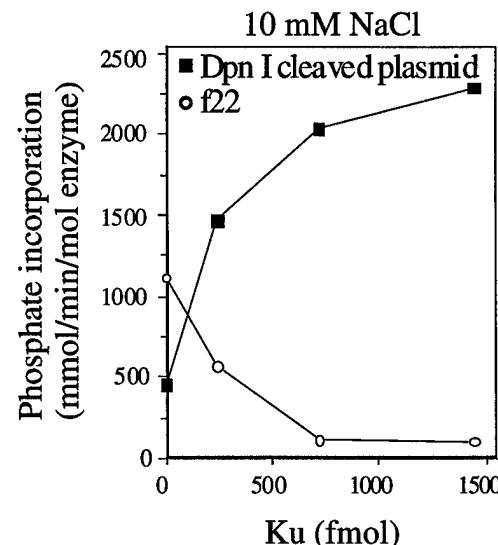
since the purified p460 was activated by the addition of DNA to phosphorylate its specific peptide substrate (27).

Is it possible that these results were caused by a small amount of Ku contamination in the preparation? Several different experiments ruled this out. First, the level of Ku contamination was extremely low, less than 1 part in 2000. We calculated that the level of p460 DNA binding activity was 100-fold too high to be explained by the most conservative estimate of the amount of Ku in the preparation. Second, the DNA binding properties of p460 and Ku were significantly different. Unlike Ku, p460 binding to f32 DNA was salt labile and competitively inhibited by both supercoiled DNA and single-stranded DNA. Third, when the p460 preparation was tested in 150 mM or 200 mM NaCl buffer, no kinase activity was observed unless purified Ku was added. Fourth, and most significantly, p460 was capable of binding to a 22 bp DNA fragment (f22), which was too short to allow the simultaneous binding of both Ku and p460 (data not shown). Since Ku has a DNA footprint of 25-30 nucleotides (19, 35), the f22 DNA was not long enough to allow binding by both Ku or p460. In a 10 mM NaCl buffer, p460 kinase activity was strongly activated by the addition of f22 (Fig. 3). Subsequent addition of Ku actually inhibited the kinase activity of p460, demonstrating conclusively that the activation of p460 kinase does not require Ku. Together, these experiments rule out any confounding role for Ku contamination. Furthermore, they demonstrate that p460 is capable of acting as a self-contained kinase that is activated by binding directly to DNA.

If the kinase function is completely contained in p460, what then is the role of Ku? Although p460 bound to f32 in 10 mM NaCl, the binding activity and kinase activity disappeared at 100 mM and 200 mM NaCl. The addition of Ku stabilized DNA binding and restored kinase activity in the higher salt concentrations. The addition of Ku had a marked effect in stimulating the p460 kinase activity when linearized plasmid DNA or Dpn I cleaved plasmid was used for activation (Fig. 3). Since the DNA binding of p460 is salt labile and competed by supercoiled plasmid DNA, the role for Ku may be to stabilize the binding of p460 to DNA ends. Although Ku is not absolutely required for the activation of the p460 kinase function by DNA ends, Ku directs p460 more efficiently to the DNA ends, particularly at physiological salt concentrations.

Figure 3. The p460 kinase is activated by DNA in the absence of Ku.

Kinase activity was measured by incubating protein with 32 P- γ -labeled ATP and a peptide containing a sequence specific for DNA-PK, EPPLSQEAFADLWKK (27). Peptide phosphorylation was completely dependent on addition of DNA ends. Kinase activity was measured for 70 fmol of p460 with the different amounts of Ku in buffer containing 10 mM NaCl. The p460 kinase was strongly activated by DNA, even in the absence of Ku. For Dpn I cleaved plasmid DNA, the addition of Ku stimulated the activation of p460. However, for f22 DNA, which was too short to allow the simultaneous binding of both Ku and p460 (data not shown), the addition of Ku resulted in the inhibition of kinase activity. This demonstrated conclusively that p460 kinase activation does not require Ku.



Gottlieb and Jackson (19) previously reported that purified p460 did not have measurable kinase activity in the absence of Ku. There are several possible explanations for the difference between their results and ours. They used linearized plasmid DNA to activate the kinase, but we found this to be a much less efficient substrate for activation than shorter DNA fragments, probably because p460 binds well to internal DNA sites as well as to DNA ends. They used small amounts of either Sp1 or HSP70 protein as the phosphorylation substrate. By contrast, we used a peptide substrate optimized for DNA-PK at concentrations 200-fold higher on a molar

basis. These differences in the length of the DNA substrate and both type and amount of the phosphorylation substrate may explain why Gottlieb and Jackson failed to detect the p460 kinase activity.

Hairpin ends will bind Ku but fail to activate DNA-PK

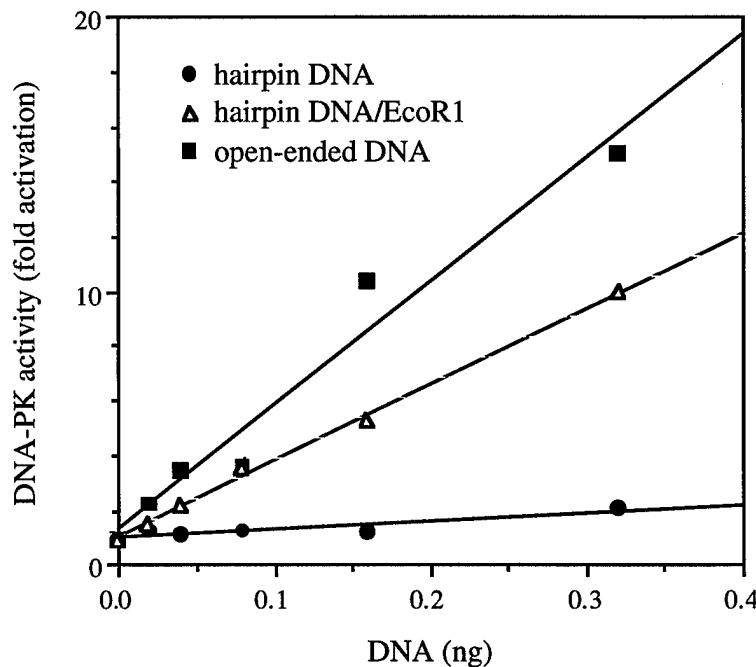
The lymphoid specific proteins RAG1 and RAG2 initiate V(D)J recombination by recognizing recombination signal sequences adjacent to V, D, or J coding sequences and catalyzing a coordinated cleavage at two sites, each at the border between a signal sequence and a coding sequence (33, 60). Cleavage occurs in a two step reaction, first nicking the DNA to leave a 3'-hydroxyl and 5'-phosphate, and then mediating a nucleophilic attack of the 3'-hydroxyl on the opposite strand to form a covalently closed hairpin coding end and a blunt signal end.

Following cleavage, joining of the DNA ends requires DNA-dependent protein kinase (DNA-PK). Ku, the regulatory subunit, binds to DNA ends to direct the activation of p460, the catalytic subunit. *In vivo*, the mouse scid mutation in p460 disrupts joining of the hairpin coding ends but preserves significant joining of the open signal ends (31).

To better understand the role of DNA-PK in V(D)J recombination, a DNA molecule with perfect hairpin ends was synthesized from 3 oligonucleotides in a collaboration with Dr. Susanna Lewis, at University of Toronto. Two of the oligos contained palindromic sequences to allow the formation of the hairpin ends, and the third oligo annealed to the overhanging ends of the other 2 oligos to form a duplex DNA molecule. This hairpin-ended DNA molecule was then tested for its interactions with DNA-PK. Surprisingly, hairpin-ended DNA was an extremely poor substrate for activating DNA-PK, producing at most 7% of the kinase activity observed for open-ended DNA of approximately the same length (Fig. 4). Although Ku binding to hairpin ended DNA was 2 to 4-fold less efficient than to open-ended DNA, this modest decrease in affinity does not appear to fully account for the failure of hairpin ends to activate the kinase. Thus, p460 may be recruited to hairpin ends in a nonfunctional form, or p460 may discriminate between hairpin and open ends.

Figure 4. Failure of hairpin ends to activate DNA-PK.

DNA-PK was incubated with a 68 bp hairpin-ended DNA molecule (closed circles), the hairpin-ended DNA cleaved with Eco RI (open triangles), or a 70 bp open-ended DNA molecule (closed squares) in the presence of peptide substrate. DNA-PK activity was measured as the fold increase in phosphorylation of the peptide in the presence of DNA over phosphorylation in the absence of DNA. The hairpin-ended DNA failed to activate DNA-PK. By contrast, DNA-PK was strongly activated by either the Eco RI cleavage products or the open-ended DNA.



We were surprised by the failure of hairpin-ended DNA to fully activate DNA-PK. Therefore, we decided to examine the effect of DNA nicks on DNA-PK, since nicks bind to Ku (3) and are created as a DNA intermediate during V(D)J recombination (33). Thus, p460 might be recruited and activated at the nicking step, so that when RAG1 and RAG2 complete the cleavage reaction, DNA-PK could assemble on the hairpin end in its activated form. We

therefore tested the activation of DNA-PK with two different nicked DNA substrates: the 68 bp hairpin molecule containing a single nick, and plasmid DNA nicked with DNase I. As observed with hairpin-ended DNA, both nicked substrates failed to activate DNA-PK, yielding no more than 7% of the activity observed with open-ended DNA.

What are the implications of these results for V(D)J recombination? DNA-PK is required for efficient hairpin processing (48, 61) but fails to be activated by hairpin ends. In addition, the kinase activity of p460 need not be intact for signal ends to be joined as seen in scid cells (4, 38), even though it is fully activated by blunt signal ends. This apparent paradox can be explained if processing of hairpin ends requires activation of DNA-PK assembled on the co-generated signal end.

We propose a model for V(D)J recombination in which DNA-PK participates in processing hairpin ends by autophosphorylating Ku and p460 in trans. In particular, Ku bound to hairpin ends would be transphosphorylated by DNA-PK bound to the co-generated signal ends. Ku has an ATPase activity that is activated by DNA-PK phosphorylation (7) and has been reported to have a helicase activity as well (58). Once activated by DNA-PK, the Ku ATPase might unwind the hairpin into a looped structure to facilitate cleavage by a putative hairpin endonuclease leading to subsequent joining of the coding ends. In this model, the signal ends are joined directly without processing, and so do not require Ku transphosphorylation. Ku bound to the signal ends might even be protected from phosphorylation by RAG1 and RAG2, which remain bound to the signal ends after cleavage *in vitro* (1). This explains the puzzling observation that signal joint formation is spared in scid mice, since Ku bound to the signal end would not be phosphorylated by p460 even in wild type mice.

The model for transphosphorylation also applies to DSB repair. Thus, Ku and p460 bound to one DNA end would be phosphorylated in trans by DNA-PK assembled on the other end. To test the model, the autophosphorylation of p460 was measured as a function of DNA-PK concentration. The autophosphorylation reaction should follow first or second order kinetics, depending on whether phosphorylation occurs in *cis* or in *trans*. In fact, the autophosphorylation of p460 followed second order kinetics (Fig. 5), demonstrating that the reaction occurred in *trans*. In a control reaction, the phosphorylation of a peptide substrate specific for DNA-PK, EPPLSQEAFADLWKK, was found to follow approximately first order kinetics, as predicted.

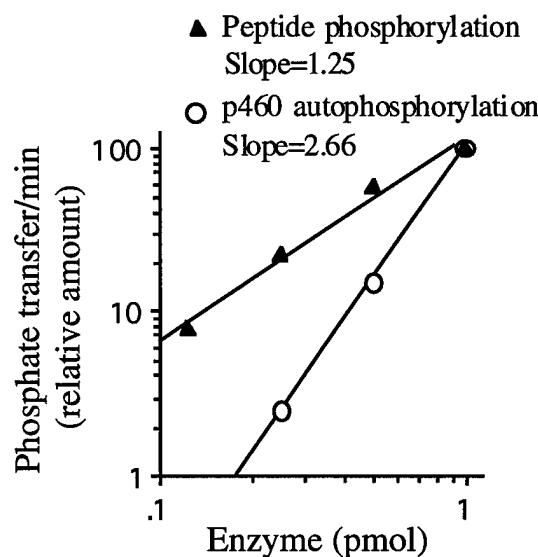
Figure 5. Autophosphorylation of p460 occurs in trans.

Ku and p460 in a 1:1 molar ratio were incubated with a molar excess of f32 in the presence of $\gamma^{32}\text{P}$ -labeled ATP. The phosphorylation of the DNA-PK specific substrate was measured in one experiment as a control for first order kinetics with respect to increasing concentration of DNA-PK. The auto-phosphorylation of p460 was measured in a second experiment. The results are plotted on a log-log scale, so that a slope of 1 corresponds to first order kinetics and a slope of 2 corresponds to second order kinetics. The slopes are in fact slightly greater than 1 and 2, perhaps because the binding of DNA-PK to DNA is somewhat unstable for a DNA fragment of only 32 bp.

CONCLUSIONS

The same biochemical pathway is involved in two distinct processes: double-strand break repair and V(D)J recombination. Therefore experiments focused on one process shed light on the other.

Double-strand break repair requires DNA-dependent protein kinase (DNA-PK), a



serine/threonine kinase that is activated by DNA. DNA-PK consists of a 460 kDa subunit (p460), which contains a putative kinase domain, and a heterodimeric subunit (Ku), which binds to double-stranded DNA ends. Previous reports suggested that DNA-PK is activated by the binding of Ku to DNA. To investigate this further, p460 and Ku were purified separately to homogeneity. Surprisingly, p460 was capable of binding to DNA in the absence of Ku. The binding of p460 to double-stranded DNA was salt labile and could be disrupted by single-stranded DNA, properties distinct from the binding of Ku to DNA. Under low salt conditions, which permitted the binding of p460 to DNA ends, the kinase was activated. Under higher salt conditions, which inhibited the binding of p460, full activation of the kinase required the addition of Ku. Significantly, when the length of DNA decreased to 22 bp, Ku competed with p460 for DNA binding and inhibited kinase activity in a dose-dependent manner. These data demonstrate that p460 is a self-contained kinase that is activated by direct interaction with double-stranded DNA. Furthermore, the DNA-PK complex is assembled by cooperative interactions among p460, Ku, and DNA.

V(D)J recombination is initiated by a coordinated cleavage reaction that nicks DNA at two sites and then forms a hairpin coding end and blunt signal end at each site. Following cleavage, the DNA ends are joined by the repair pathway that depends on DNA-PK. In vivo, the mouse scid mutation in p460 disrupts joining of the hairpin coding ends but preserves significant joining of the open signal ends. To better understand the mechanism of V(D)J recombination, we measured the activation of DNA-PK by DNA nicks and hairpin ends. Surprisingly, nicks and hairpin ends did not activate the kinase, even though hairpin coding ends require intact DNA-PK to be processed and joined. To resolve this apparent paradox, we hypothesize that hairpin ends are processed by activation of the kinase by the co-generated signal ends, and that the target for phosphorylation is DNA-PK bound to the hairpin ends. In support of this hypothesis, we have now found that the stimulation of DNA-PK by open DNA ends leads to an autophosphorylation of p460 that follows second order kinetics and therefore must occur in trans.

These experiments have dissected early steps in the pathway for DNA double-strand break repair. Since double-strand breaks are the most important lesions produced by ionizing radiation, and since exposure to ionizing radiation is a critical risk factor for breast cancer, these results will lead to progress in the prevention and treatment of breast cancer.

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